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Biological Infrared Microspectroscopy at the National Synchrotron Light Source

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Abstract. Beamline U2B at the National Synchrotron Light Source has been designed and built as an infrared beamline dedicated to the study of biomedical problems. In 1997, the horizontal and vertical acceptances of Beamline U2B were increased in order to increase the overall flux of the beamline. A wedged, CVD diamond window separates the UHV vacuum of the VUV ring from the rough vacuum of the beamline. The endstation consists of a Nicolet Magna 860 step-scan FTIR and a NicPlan infrared microscope. The spectrometer is equipped with beamsplitter/detector combinations that permit data collection in the mid- and far-infrared regions. We have also made provisions for mounting an external detector (e.g. bolometer) for far infrared microspectroscopy. Thus far, Beamline U2B has been used to (1) perform chemical imaging of bone tissue and brain cells to address issues related to bone disease and epilepsy, respectively, and (2) examine time-resolved protein structure in the sub-millisecond folding of cytochrome c.

BEAMLINE DESIGN AND CHARACTERIZATION

The high brightness of a synchrotron infrared (IR) source dramatically improves the spatial resolution of an IR microscope, such that data can be obtained at the diffraction limit, which is 3-5 μm in the mid-IR region (1-5). Beamline U2B at the National Synchrotron Light Source (NSLS) has been designed and built as a beamline dedicated to the study of biomedical problems. In 1997, the opening angle of Beamline U2B was increased to 40 x 40 mrad. The beam is extracted from the ring by a water-cooled, gold-coated silicon plane mirror. A wedged, CVD diamond window separates the UHV vacuum of the VUV ring from the rough vacuum of the beamline. The beamline endstation consists of a Nicolet Magna 860 step-scan FTIR and a NicPlan IR microscope, both of which are purged with dry nitrogen gas. The rough vacuum of the beamline is isolated from the purged spectrometer and microscope by a KBr or polyethylene window. The spectrometer is equipped with interchangeable (1) KBr and solid substrate™ beamsplitters and (2) MCT and DTGS detectors, which permit data collection in the mid- (650-4000 cm^{-1}) and far- (10-650 cm^{-1}) IR regions. The NicPlan IR microscope is equipped with an MCT detector, a video imaging camera, and motorized x-y sample stage. We have also made provisions for mounting an external detector (e.g. bolometer) for far IR microspectroscopy.

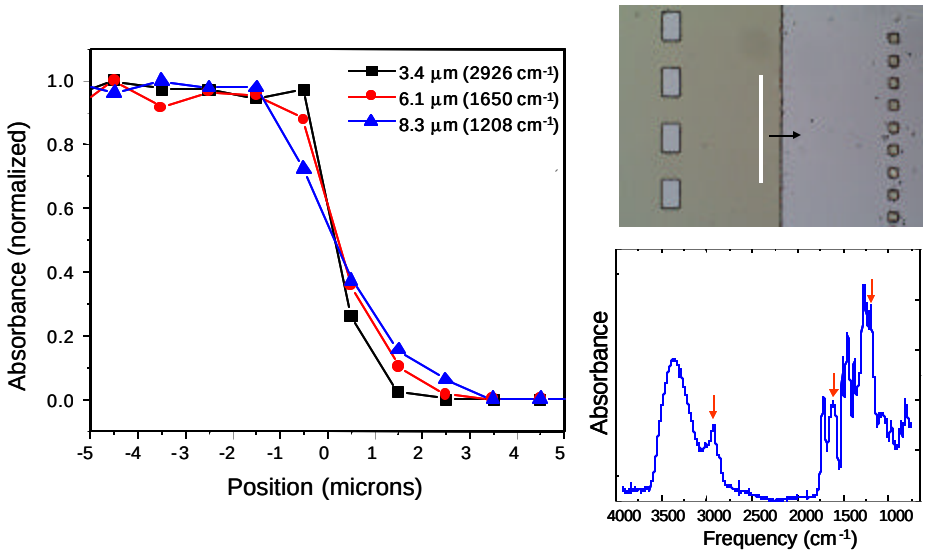

FIGURE 1. Spatial resolution characterization of Beamline U2B. *(Top Right)* A 1 x 60 µm aperture is scanned in one micron steps across a photoresist pattern edge. *(Bottom Right)* IR spectrum of the photoresist film. *(Left)* IR absorbance is determined at each point for three absorbance features in the photoresist spectrum: 2926 cm^{-1}, 1650 cm^{-1}, and 1208 cm^{-1}. These frequencies were chosen because they fall close to that of lipids, proteins, and nucleic acids, respectively, all of which are components of biological samples.

The spatial resolution of the IR microscope has been characterized experimentally and can be observed in Figure 1. A 1 x 60 µm aperture was set on the microscope and scanned across the edge of a photoresist pattern deposited on a gold-coated microscope slide (Spectra Tech, Inc.). The experiment was performed in reflection mode, to take full advantage of the confocal (dual aperture) nature of the microscope while avoiding any dispersion effects from transmission measurements through the photoresist pattern on a BaF_2 substrate (5). Absorbance values were recorded for three features in the photoresist spectrum as a function of position across the edge. From Figure 1, it can be seen that the step edge across the photoresist pattern is sharper for the 3.4 µm feature (~ 1.5 µm) than the 8.3 µm feature (~ 4 µm). These results demonstrate that spatial resolution is frequency-dependent, where the spatial resolution at shorter wavelengths is better than longer wavelengths.

BIOLOGICAL CELL AND TISSUE CHEMICAL IMAGING

The improved spatial resolution obtained from a synchrotron IR source opens the door to numerous biological applications that are not possible with a conventional globar source. The average size of a biological cell is 10-30 µm in diameter. With a thermal source, only a single spectrum can be obtained from that cell. However, the synchrotron source permits chemical imaging of living cells, which has been demonstrated on both mitotic (dividing) and dying cells (6, 7). In addition, IR microspectroscopy at the NSLS has been used to examine the chemical composition of various diseased states of bone (8-10). In the osteoporosis experiments, the effects of estrogen deficiency on the composition of bone have been studied by combining

fluorescence microscopy and IR microspectroscopy. Figure 2 contains a fluorescence microscope image of monkey cortical bone. The bright areas designate fluorochrome-labeled bone, which is new bone deposited after the monkey's ovaries are removed, i.e. after the onset of estrogen deficiency. The IR spectra in Figure 2 demonstrate that the older, mature bone is more mineralized (spectrum #1) than the fluorochrome-labeled bone (spectrum #2), as evidenced by increased phosphate absorption between 900-1200 cm^{-1} and 500-650 cm^{-1}.

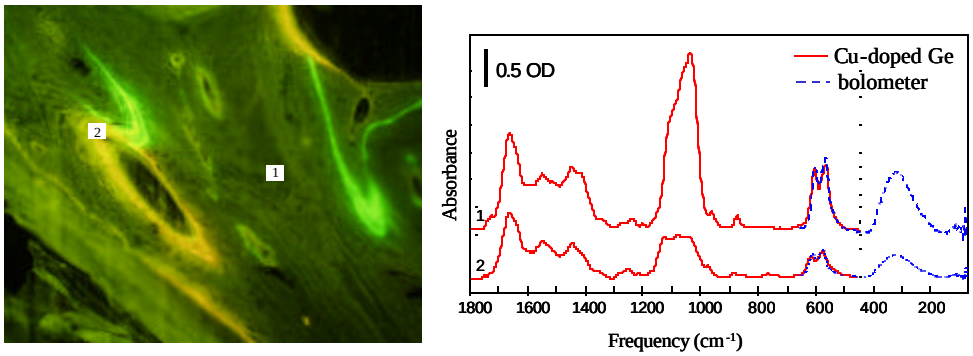

FIGURE 2. (Left) Fluorescence microscope image of monkey cortical bone. Bright areas indicate fluorochrome-labeled bone. Boxes represent 10 x 10 μm apertures used to collect IR spectra. (Right) IR spectra collected from unlabeled and labeled bone, respectively, 128 scans, 4 cm^{-1} resolution. Data were collected with a Cu-doped Ge detector and bolometer.

The interchangeable beamsplitters on Beamline U2B provide the added capability of collecting IR microscope data in the far IR for the first time. We use a polyethylene window on the beamline, a solid substrate™ beamsplitter, and we have made provisions for mounting an external detector (e.g. bolometer) on the Nic Plan microscope. The FTIR spectrometer, microscope, and detector area are all sufficiently purged with dry nitrogen. Figure 2 illustrates the first IR microscope spectra collected on a biological sample (bone) in the far IR. In addition to the $\nu_4PO_4^{3-}$ region (500-650 cm^{-1}), we also observe a broad, collagen band centered near 350 cm^{-1}.

BIOMOLECULAR STRUCTURE AND DYNAMICS

In addition to cellular imaging, the examination of bio-molecule structural dynamics can also benefit from the high brightness of the synchrotron IR source. We have developed a sub-millisecond, continuous-flow mixer for studying reaction dynamics. Conventional mixers have dead times in the millisecond time regime. However, the micro-mixer we describe here takes advantage of the small, bright spot size of the synchrotron source and dramatically reduces both mixing dead time and required sample volumes (Figure 3).

In one experiment, we are examining the protein structure of cytochrome c during the folding process. It is well-established that the Amide I band of a protein IR spectrum is sensitive to structure (11, 12). Cytochrome c is an iron-containing protein found in the respiratory chain. It is primarily α-helical in the folded state, but exists in

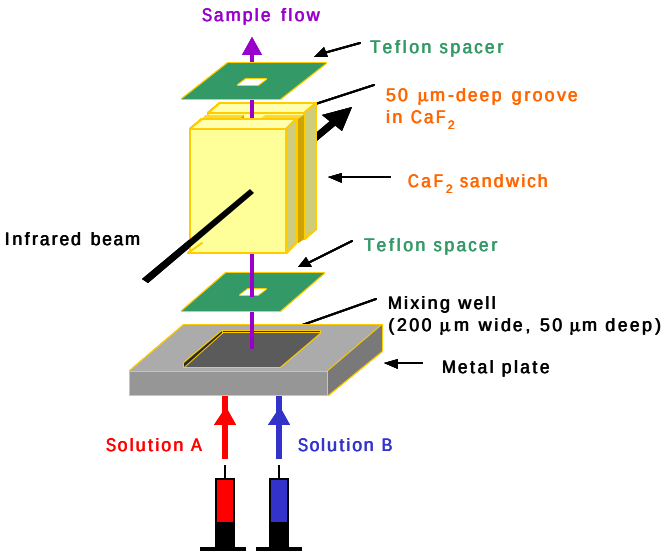

FIGURE 3. Time-resolved IR spectroscopy flow cell. For protein folding experiments, solution A is a folded (or unfolded) protein in D_2O buffer and solution B is a denaturant (or renaturant) in D_2O. The solutions flow into a tiny mixing chamber at a fast flow rate, so that the turbulence mixes the solutions with a sub-millisecond dead time. The mixed solution then flows into a CaF_2 IR sandwich cell containing an etched groove. The entire apparatus is mounted on the stage of the IR microscope. IR spectra at various time points after mixing are collected by translating the IR beam along the channel in the flow cell.

a random coil conformation in the unfolded state. We are using the rapid-mixer to elucidate the pathway by which the protein folds from its unfolded to folded state.

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REFERENCES

1. Reffner, J. A., Martoglio, P. A. and Williams, G. P., *Rev. Sci. Instr.* **66**, 1298, (1995).
2. Carr, G. L., Reffner, J. A. and Williams, G. P., *Rev. Sci. Instr.* **66**, 1490-1492, (1995).
3. Carr, G. L. and Williams, G. P., *SPIE Conf. Proc.* **3153**, 51-59, (1997).
4. Carr, G. L., *Vibrational Spectroscopy* **19**, 53-60, (1999).
5. Carr, G. L., Merlo, O., Munsli, M., Springer, S. and Ho, S. C., *SPIE Conf. Proc.*, in press.
6. Jamin, N., Dumas, P., Moncuit, J., Fridman, W. H., Teillaud, J. L., Carr, G. L. and Williams, G. P., *Proc Natl Acad Sci U S A* **95**, 4837-40, (1998).
7. Jamin, N., Dumas, P., Moncuit, J., Fridman, W. H., Teillaud, J. L., Carr, G. L. and Williams, G. P., *Cell Mol Biol (Noisy-le-grand)* **44**, 9-13, (1998).
8. Miller, L. M., Carlson, C. S., Carr, G. L. and Chance, M. R., *Cellular and Molecular Biology* **44**, 117-127, (1998).
9. Miller, L. M., Huang, R., Chance, M. R. and Carlson, C. S., *Synchrotron Radiation News* **12**, 21-27 (1999).
10. Miller, L. M., Carlson, C. S., Hamerman, D. and Chance, M. R., *SPIE Conf. Proc.*, in press.
11. Susi, H. and Byler, D. M., *Biochem Biophys Res Commun* **115**, 391-7, (1983).
12. Byler, D. M. and Susi, H., *Biopolymers* **25**, 469-87, (1986).